Individual variation in corticosterone and personality traits in the blue tit *Cyanistes caeruleus*

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Summary

Variation in personality traits is predicted to reflect physiology, but the extent to which variations in stress hormones derive from differences in personality and/or state-dependent factors remains unclear. To investigate this, wild blue tits *Cyanistes caeruleus* were briefly brought into captivity and scored for personality and corticosterone (Cort) concentrations. More active females had lower baseline Cort than less active individuals. Exploratory tendency and neophobia did not co-vary with baseline Cort. Stress-induced Cort concentrations were correlated negatively with exploratory tendency and haematocrit, but positively with mass gain in captivity. Baseline and stress-induced Cort concentrations in wintering blue tits were associated with state-dependent variables sex, age and personality traits. Key to interpreting the physiology of personality traits seems to be their interactions with other traits that mediate ability to utilise resources, and thus influence an individual's perception of its current and future energy balance.

Introduction

When exposed to the same environmental or social stimuli, under standardised conditions, the behavioural (Verbeek et al., 1996; Gosling, 2001) and physiological responses (Cockrem, 2007) of individuals of the same species often differ. Behavioural differences that are stable within individuals measured repeatedly across a range of situations or contexts are often referred to as 'personality traits' (Gosling, 2001). While the heritability of personality traits (Drent et al., 2003; Oers et al., 2004; Oers et al., 2005; Martins et al., 2007) suggests that they have a genetic basis, they can also be affected by early environmental conditions (e.g. (Arnold et al., 2007; Stamps & Groothuis, 2010). Similarly, both genes and early environmental conditions have been shown to affect physiological systems such as the stress axis of adults (Monaghan, 2008; Koolhaas et al., 2010). The consistent nature of these differences between individuals suggests they may contribute towards variability in fitness-related traits among individuals (Buchanan, 2000; Both et al., 2005; Blas et al., 2007; Bonier et al., 2009).

In birds, the physiological stress response is modulated by both basal levels and the acute release of the adrenal steroid corticosterone (Cort), amongst other hormones. Basal concentrations of Cort have been reported to vary in response to environmental variables such as weather (Astheimer et al., 1995; Rubenstein, 2007) food availability (Jenni-Eiermann et al., 2008), as well as state-dependent variables such as an individual's body condition (Raja-aho et al., 2010) or health status (Martin, 2009). These changes in basal Cort can trigger a series of physiological and behavioural changes that should restore homeostasis. For example the increase in Cort seen following food restriction simulates increased motivation to feed (Lohmus et al., 2003; Long & Holberton, 2004; but see Piersma et al., 2000). Chronic elevations of basal Cort, due to long term or repeated exposure to a stressor, have been reported to have negative effects, suppressing insulin production hence rendering the animal prone to fatigue and disease (reviewed in Buchanan, 2000, see also (Goutte et al., 2010Hau & Goymann, 2015). However, high basal Cort might not necessarily

signal challenging conditions: basal Cort also varies consistently across individuals under the same conditions, with some individuals showing relatively high and others showing low levels of Cort. The extent to which such differences in basal Cort reflect individual differences in personality and fitness are still the focus of discussion (Bonier et al., 2009; Angelier et al., 2010; Dingemanse et al., 2010; Koolhaas et al., 2010; Hau & Goymann, 2015).

Rapid on-set, acute stress responses evoked in response to an 'emergency' often involve an immediate increase in blood glucose, to facilitate rapid behavioural reactions such as fight or flight (Cockrem & Silverin, 2002a) and are generally considered to be adaptive (Buchanan, 2000). In Parids, like many small passerines (Wingfield & Kitayskya, 2002), the acute stress response is generally characterised by a rise in Cort that usually peaks between ten and approximately 40 minutes after the stressor before declining (Cockrem & Silverin, 2002b; Henderson et al., 2014)). The level and timing of the peak in Cort varies both within and between species (Bokony et al., 2009; Cockrem et al., 2009; Henderson et al., 2014). Extensive blood sampling is not possible in small birds, so researchers generally present data on 'stress-induced' (rather than strictly peak) Cort concentrations, measured after an animal has been held for a set time under standardised restraint conditions (Wingfield, 1994). As physiological responses to stress are often accompanied by changes in behaviour, there is a prediction, with some supporting data, that personality types might show associated consistent differences in both basal and acute Cort levels (Cockrem, 2007; Hau & Goymann, 2015). These effects might be directly related, for example more neophobic juvenile rats have a higher glucocorticoid response as adults (Cavigelli & McClintock, 2003), or indirect, for example where personality affects the ability to acquire or control food, which might have an impact on both stress-induced and baseline Cort (Koolhaas et al., 2010). Similarly, age, sex and dominance status can affect resource holding potential and thus energetic balance (Gosler, 1996); Fat reserves reflect a trade-off between starvation (high fat reserves are beneficial) and predation (high fat reserves slow down escape flight, so are costly) resulting in great tits *Parus major* living under high perceived predation risk carrying lower fat reserves than under low perceived predation risk.

Moreover, dominant birds (adult males) carried the least fat, presumably because they were able to displace subordinate birds (juvenile females) at feeders, so had a low risk of starvation despite their low fat reserves (Gentle & Gosler, 2000). Thus, age, sex and dominance status might also influence both baseline and stress-induced Cort (Rubenstein, 2007). So although there is evidence of correlations between an individual's personality and its glucocorticoid profile (Carere et al., 2003; Cavigelli & McClintock, 2003; Cockrem, 2007; Martins et al., 2007; Cavigelli et al., 2009; Koolhaas et al., 2010), causality is difficult to assign.

Assessments of 'personality traits' in animals result in individuals being traditionally defined as falling somewhere on a linear continuum: neophobic-neophilic or bold-shy (Wilson et al., 1994); aggressive-passive; (Huntingford, 1976); active-inactive (Sih et al., 1992) and fast-slow explorers (Verbeek et al., 1994). Such personality traits are often highly correlated within individuals, for example activity and exploration (Dingemanse et al., 2007; Martin & Reale, 2008), or boldness and aggression (Bell, 2005). A commonly described trait correlation is the "proactive-reactive" syndrome (Carere, 2003; Koolhaas et al., 2007). Such correlations imply proximate links between traits, via genetic linkage or shared physiology (Verbeek et al., 1994). In a review, Cockrem (2007) concluded that across avian species, birds with 'proactive' (bold, fast exploring, aggressive etc.) personalities have relatively active behavioural responses and low stress-induced Cort concentrations, whilst birds with reactive personalities have relatively passive behaviour and large stress-induced Cort concentrations. However, correlations can also occur between traits that are not mechanistically connected but rather subject to the same selection pressures or jointly determined by other factors (Bell & Sih, 2007; Dingemanse et al., 2007; Ruiz-Gomez et al., 2008; Carere et al., 2010). Furthermore, not all species show correlations between such suites of behaviours (e.g. (Bell & Sih, 2007; Herborn et al., 2010; Herborn et al., 2011) which would suggest that the regulation of these traits may differ between species. Thus, while neophobia and exploratory tendency are correlated in many but not all species (Herborn et al., 2010) they could differ in their associations with baseline and stress-induced Cort concentrations within individuals and across populations. This is in line with

Cockrem et al.'s (2009) assertion that there is no optimum Cort response for all conditions that maximises fitness.

Whether or not an individual's hormonal stress response is a function of environmental and state-dependent variables or associated with personality is important for understanding the mechanisms underlying fitness variation. In this study, we investigated the environmental, state-dependent and behavioural factors that might be associated with inter-individual differences in baseline and stress-induced Cort in wild blue tits *Cyanistes caeruleus*. In contrast to studies of the closely related great tit (Verbeek et al., 1996), we have previously shown that neophobia and exploratory tendency are uncorrelated in blue tits (Herborn et al., 2010). This would suggest that in the blue tit these traits can potentially differ in their relationships with basal and/or stress-induced Cort. Moreover, we have found that personality types differed in antioxidant defences, and it was the combination of an individual's personality traits that proved important (Arnold et al., 2015). This suggests that the physiology underlying personality traits is complex. Here, we specifically addressed whether an individual's baseline or stress-induced plasma Cort concentrations were correlated with: 1) weather variables on the day of capture, as these would affect opportunities to forage and thus metabolic state; 2) personality traits (neophobia and two measures of behaviour in a novel environment: exploratory tendency and activity); 3) state-dependent variables (condition, haematocrit, change in mass in captivity and motivation to feed) and permanent inter-individual differences (or variables that would not change within a season i.e sex, age and size), as these may also affect foraging opportunities in the wild and therefore hormonal status.

Material and methods

The study was conducted over two winters between 2007 and 2009 on a population of blue tits living in oak dominated woodland on the east bank of Loch Lomond, UK (56°08'N 4°37'W) (Arnold et al., 2007). In October 2007, eight feeding stations were established at approximately 500m intervals

(Herborn et al., 2010). These feeding stations were removed at the end of February 2008 and reinstalled in the same positions between October 2008 and February 2009. Between November and February, birds were captured as they approached the feeding stations, using mist-nets. Mist-netting was conducted between dawn and noon, three times at each feeding station in the winter of 2007-8 and twice in 2008-9. Birds arrived in captivity generally between 10:00 and 12:00, within 15 minutes journey time from their capture site. A total of 125 blue tits were caught, with 69 birds (54 male: 15 female) for which we had a Cort sample and a full set of behavioural data being included in this study. For the other 56 individuals, in most cases we did not have a large enough blood sample to run the Cort analyses as well as other planned blood assays, in a few cases the bird did not perform properly in one of the behavioural trials so had to be excluded and/or a morphometric measurement was missing. This sex ratio bias reflected that of the population of birds caught at the feeders (Herborn et al., 2010). Birds were then taken into captivity for 2 days in 2007/8 and 3 days in 2008/9. In 2007/8 roughly two hours after blood sampling, birds were then released at the site of capture. In 2008/9, birds were released on day 3 following the second exploration trial.

Environmental variables on the day of capture

Variables that may have affected foraging immediately prior to capture and hence metabolic state were recorded. These included day length, rainfall (mm) and minimum and maximum temperature for the day of capture. Weather data were collated from records for Glasgow Bishopton sent to us by the Met office.

Personality traits

After removal from the mist-net, birds were held in cloth bags and transported, up to 3km, to the Scottish Centre for Ecology and the Natural Environment. After arrival birds were scored for their morphometrics and housed indoors, at a temperature of 17°C±1°C on a 12:12 hour light:dark cycle (to enable us to conduct all of the tests and minimise the time the birds would be held in captivity

whilst standardising conditions across birds) . Trials ran from mid-November until the end of January, during which time the natural daylength was at maximum 8hr30 and minimum 7hr. Each bird was housed individually in a 150cm x 50cm x 50cm high cage, with six cages per room arranged such that birds were visually but not acoustically occluded from one another. Birds were fed peanut granules, Haiths' Prosecto™ insectivorous mix and water *ad libitum*, supplemented with around ten *Tenebrio molitor* and two *Galleria mellonella* larvae per day. All birds were observed eating within 10 minutes of arrival in captivity. After arrival birds were left undisturbed for a minimum of 2 hours and a maximum of 3 hours before the start of personality trait assessments. As we wanted to return the birds to the wild as soon as possible, we did not allow the birds a long acclimation period in captivity. We know that the personality traits measured in captivity predicted neophobia and exploratory tendency measured for the same individuals in the wild weeks or even months later (Herborn et al. 2010). So we have some evidence that the short acclimation period was not having significant negative impacts on our behavioural assays. Three behavioural traits, exploratory tendency, activity and neophobia were assayed as fully described in Herborn et al (2010):

Exploratory tendency was assessed within what would become the home cage of the focal bird. Each cage was split into two halves with an opaque cage divider (Figure S1 in Electronic Supplementary material (ESM)). On arrival into captivity, the bird was introduced to one side of the cage, selected at random, and allowed to acclimate for two hours thereby creating a "familiar" and, behind the divider, a "novel" environment. To assay exploratory tendency and not neophobia, the arrangement of plastic plants and perches was the same in each cage half, so that the novel environment was novel only in that it was unexplored. At the start of the trial, the food bowl and any spilt food were removed and 30 mins after that the water bowl was also removed, this was to ensure that the birds did not spent the whole trial eating or drinking, thus making it difficult to determine their motivation for moving. After a further 30 mins, the researcher removed the opaque cage divider and observed the focal bird for 10 mins. Unlike other exploration trials (e.g. (Verbeek et al., 1994), individuals had the option of remaining within the familiar environment. This allowed us

to distinguish activity due to exploration which is related to information gathering about a novel environment, from activity due to escape behaviours which is not necessarily linked to information gathering (Mettke-Hofmann et al., 2009).The number of movements (hop or flight) in each side of the cage was recorded, with the endpoint of each movement defining the side of the cage: novel or familiar. Exploratory tendency was calculated as the number of movements in the novel environment minus the number of movements in the familiar environment. In 2008/9, the exploratory tendency trial was repeated between 08:00 and 011:00 on day 3 of captivity before releasing the birds. This was to allow us to calculate the repeatability of this behavioural trait. To create a new novel environment, the paper lining the cage was changed from white to brown or vice versa. Also, the arrangement and size of perches and artificial plant material were similar between these cage types, but different leaf shapes were used in the brown versus white-lined cages.

Activity was derived from the same exploration trial, as the number of movements in both the familiar and novel side of the cage. We described behaviour in the exploration trial in these two ways to determine whether patterns relating to exploration were due to individual variation in true exploratory tendency, as reflected by space use within the trial, or simply activity levels, as commonly reported in other exploration studies (Verbeek et al., 1994; Martins et al., 2007).

Neophobia was assessed over two trials conducted between 13:00 and 17:00 on day 1 and 08:00 and 11:00 on day 2. Each trial had two phases: a novel object phase and a disturbance control phase. Food and water were removed for 30 mins prior to each phase. Water was removed so that during the experiment only one resource, food, was available. In the novel object phase, the observer then returned the food bowl with one of two novel objects (different novel objects were used in the two trials) placed inside. The objects were a bright pink plastic frog and half of a purple rubber ball, of similar size (approximately 4cm diameter and 2cm height) (Figure S2 in ESM). The latency to land on familiar food bowl was recorded. The object was then removed and the water returned. The disturbance control phase was completed so as to allow interpretation of the results relative to any possible differences in an individual's motivation to feed, or their response to

disturbance by the observer returning the food bowl to the cage (van Oers et al., 2005). Thus in a random order either one hour before or after the novel object phase, the latency to feed was measured by food bowl removal and replacement as before but without a novel object. Neophobia was defined as the latency in the novel object phase minus the latency in the disturbance phase. Mean latency to approach the food bowl was significantly greater when a novel object was present (Wilcoxon Signed ranks Z = - 3.34, N = 68, p = 0.001), than when absent. Thus the presence of the novel object modified behaviour so could be said to induce neophobia.

Repeatability of neophobia, exploratory tendency or activity scores between day 1 and day 2 was calculated using the mean squares from an analysis of variance, following Lessells and Boag (1987). Neophobia across days 1 and 2 (ANOVA; r = 0.29, $F_{63, 68}$ = 1.811, p = 0.009), as well as exploratory tendency (r = 0.27, $F_{36, 68}$ = 1.71, p = 0.04) and activity (r = 0.42, $F_{36,68}$ = 2.56, p = 0.001) across days 1 and 3 were repeatable. In the following results, only data from the first of each trial per individual were analysed as they were therefore considered representative of the individual's personality traits but ensured no carry-over effects through learning or habituation to the experiments. This also meant that we had a larger sample size for exploratory tendency and activity than if we had taken a mean of both trials.

State-dependent and permanent inter-individual differences

Next, we determined whether state-dependent (i.e. changeable within a season) variables, and permanent inter-individual differences (fixed at least within one season) predicted an individual's Cort concentration. An individual's state was assessed as its body condition (residual of mass at blood sampling against tarsus length), haematocrit and change in mass in captivity (mass at blood sampling after 24 hours of captivity – mass at capture).

The duration of the first and second feeding bouts in each disturbance trial (see below) were taken as a measure of hunger and motivation to feed. Neither of these measures differed significantly between the two years, juveniles and adults or between the sexes (Mann-Whitney U-

test $p > 0.11$ in all cases), so the data were pooled. The duration of the two feeding bouts were correlated, i.e. consistent, within individuals (Kendall's tau b = 0.23, N = 56, p = 0.027), so from the first disturbance trial we analysed just the duration of the second feeding bout as an index of hunger and motivation to feed in case there was any disturbance associated with returning the bowl to the cage in the first feeding bout.

Permanent variables (unchanged within a field season) were also assessed and recorded for each bird. Wing length was used as a measure of overall body size (not measured in one bird). Age (juvenile/adult) was determined from plumage traits (Svensson, 1992). Sex was determined from a blood sample using primers P2 and P8 (Arnold et al., 2007).

Blood sampling

Approximately 24 hours after capture, birds were blood sampled. Birds were sampled after undergoing behavioural assays as there is some evidence that blood sampling and/or associated handling can change the physiology and behaviour of birds (e.g. (Le Maho et al., 1992; Cockrem et al., 2009; Dickens et al., 2009). Moreover, in captivity all the birds had received *ad libitum* food, constant temperature and light/dark cycles and minimal social interactions, thus many of the environmental factors known to affect Cort concentrations had been standardised across individuals (Romero et al., 2000; Pravosudov et al., 2001). During blood sampling, two birds per room were removed from their cages with the order randomly assigned. For baseline Cort, birds were blood sampled within 3 mins of the researcher entering the aviary room, which is generally considered to be representative of baseline Cort concentrations (Romero & Reed, 2005). For the stress-induced Cort, the bird was put into a cloth bag upon capture, removed after 14 mins and blood sampled 15 mins after initial disturbance. Caged birds were then left undisturbed for one hour before re-entry to each room to collect samples from different birds. The last birds to be sampled had therefore been disturbed a maximum of twice that day, but disturbance frequency did not significantly influence either baseline (ANOVA $F_{2,35} = 0.065$, $p = 0.94$) or stress-induced Cort (ANOVA $F_{2,29} = 0.092$, p =

0.91). To collect the blood, a sterile 25 gauge needle was used to prick the brachial vein. Blood samples were collected in 75 µl heparinized capillaries and centrifuged for 5 minutes at 14,000 g. A haematocrit score (a measure of anaemia) was taken for each tube before the red cells and plasma were split, red cells mixed with ethanol and samples stored at -70 ºC until assayed. Less than 10% of the circulating blood volume was collected, as defined by the UK Home Office. Collecting this volume of is not considered to have negative impacts on the welfare of a bird. As we could only take enough blood from each bird for a single Cort assay, due to ethical considerations and the limits defined by the UK Home Office, we only have either baseline or stress-induced Cort concentration from each individual.

Corticosterone assays

Corticosterone concentrations were measured after extraction of 5-20µl aliquots of plasma in diethyl ether, using a double antibody radioimmunoassay (Wingfield et al., 1992). Primary antibody Esoterix B183, Secondary antibody Sigma goat anti-rabbit and [3H]-corticosterone label (GE Healthcare, UK). See Henderson et al. (2013) for full methods. Extraction efficiency was 93 ±8% (mean ± SD). Recoveries were measured for samples independently and individual corticosterone concentrations adjusted accordingly. The assay detection limit was 0.03 ng/ml. Samples were run within two assays. Inter- and intra-assay coefficients of variation were 6.68% and 4.35±0.001% respectively.

Ethical Note

All work was carried out in accordance with ASAB/ABS's guidelines for the treatment of animals in research. Work was under license of the UK Home Office and subject to ethical review by WALTHAM® Centre for Pet Nutrition and the University of Glasgow. Captive studies were completed and feeders removed 2 months before the first record of nest building in the area. Birds had on average gained (2.97% ± 7.3%) body mass whilst in captivity. 108 out of the total 125 birds released were later recorded using the feeders or were re-trapped in the area.

Statistical Methods

Analyses were carried out using SPSS (Ver 20). Prior to analysis, data were checked for normality, homogeneity of variance and co-linearity. Where these assumptions were violated, data were transformed or non-parametric tests were carried out. The distribution of stress-induced Cort concentrations, for example, showed a slight skew and a log transformation improved the situation. In addition, Cook's distances were calculated for all co-variates and subsequently neophobia data from three individuals and exploration data from one individual were excluded as extreme outliers that would have distorted the outcome of the analyses. Exploratory tendency (Shapiro–Wilks test: $W_{120} = 0.94$, $p < 0.0001$), activity during the exploration trial ($W_{120} = 0.95$, $p < 0.0001$) and neophobia (Shapiro–Wilks test: $W_{78} = 0.89$, $p = <0.0001$) were leptokurtic and it was not possible to normalise their distributions. Exploration scores (Mann-Whitney U-test Age: $z = -0.38$, $N = 64$, $p = 0.71$; sex: $z = -0.23$, $N = 60$, $p = 0.82$), activity levels (Age: $z = -0.39$, $N = 64$, $p = 0.70$; sex: $z = -0.94$, $N = 60$, $p = 0.35$) and neophobia scores (Mann-Whitney U-test Age: $z = -1.25$, $N = 63$, $p = 0.21$; sex $z = -0.88$, $N = 59$, $p = 0.38$) did not differ between adults and juveniles or males and females, therefore data were pooled to analyse other sources of between-individual variation. With the Bonferroni correction threshold p-value of 0.004, all other morphometric (mass at capture, mass at blood sampling, wing length) and environmental variables (rainfall, minimum temperature and maximum temperature on day of capture) were non-significant in relation to neophobia, exploratory tendency and activity. Therefore analyses of these traits were calculated using actual scores, rather than, for example, residuals or transformed data.

General linear models (GLMs) were run with Cort plasma concentration (basal or peak) as the dependent variable, sex and age (adult or juvenile) as factors, and change in mass in captivity (mass on day 2 – mass on day1), wing length, haematocrit and personality traits as co-variates. The interactions between sex or age and personality traits or change in mass in captivity were also included in the full model. All tests were two-tailed. Means ± S.E. are presented.

Results

Neither basal nor stress induced Cort concentrations differed significantly between years (Mann-Whitney U-test z = -0.36, N = 69, p = 0.72) so data were pooled. Baseline Cort concentrations varied among individuals, from 0.33 – 13.21 ng/ml. Stress-induced plasma Cort concentrations were significantly higher (mean = 10.55 ng/ml ± 0.94, range from 1.32 – 23.83 ng/ml) than baseline concentrations (mean = 4.17 ng/ml ± 0.55; GLM $F_{1, 66} = 30.81$, $p < 0.0001$), although we did not have both measures from the same individual.

Environmental variables on the day of capture

Neither baseline nor stress-induced Cort concentrations were significantly related with daily rainfall (Baseline: Kendall's tau = 0.13, N = 38, p = 0.27; Stress-induced: Kendall's tau = 0.07, N = 28, p = 0.63), minimum daily temperature (Baseline: Kendall's tau = 0.02, N = 38, p = 0.87; Stress-induced: Kendall's tau = -0.04, N = 28, p = 0.75) or maximum daily temperature on the day of capture (Baseline: Kendall's tau = 0.00, N = 38, p = 1.0; Stress-induced: Kendall's tau = 0.06, N = 28, p = 0.63).

Personality traits

As mentioned above, we only used data from the first of each trial per individual. In these first trials, neophobia scores varied from -117s to 597s with a mean of 85.59s ± 19.18 (six birds scored zero and six birds failed to complete the trial), with positive scores indicating highly neophobic birds and negative scores meaning that the birds were not neophobic. Exploratory tendency ranged from -280 to 226 with a mean of 9.69s ± 11.79 (three birds scored zero and two birds failed to complete the trial) and total activity level from 0 – 467 with a mean of 233.42s ±15.90 (two birds scored zero, i.e. did not move, and two failed to complete the trial). High scores indicated highly exploratory and active birds respectively.

Neophobia scores were not correlated with either exploratory tendency (Kendall's tau = -0.04, N = 62, p = 0.69) or activity levels during the exploration trial (Kendall's tau = 0.01, N = 62, p = 0.89). Activity levels and exploratory tendency were uncorrelated (Kendall's tau = 0.051, N = 67, p = 0.54). Thus, exploratory tendency, activity levels during the exploration trial and neophobia are distinct personality traits (i.e. were repeatable across time), not simply three measures of the same trait or by-products of differences in activity levels. Also, we did not find evidence of a behavioural syndrome.

Baseline Cort concentrations were significantly related to the interaction between sex and activity ($F_{1, 36}$ = 5.46, p = 0.026; sex $F_{1, 36}$ = 8.82, p = 0.006, activity level $F_{1, 36}$ = 10.09, p = 0.003;Fig. 1a). Specifically, more active birds had lower baseline Cort than inactive birds. The slope of this relationship was much steeper for females than males. Using a backwards stepwise process, non-significant interactions then age, exploratory tendency (Fig. 1b) and neophobia (Fig. 1c) were removed from the model. The sample size for females in this analysis was only six, so we repeated the analysis just for males. For males there was no significant correlation between activity levels and baseline Cort (Pearson correlation = -0.244, N = 31, p = 0.185).

Stress-induced Cort was uncorrelated with activity ($F_{1, 28}$ = 0.02, p > 0.8; Fig. 1d). There was a non-significant tendency for more neophobic birds to have lower stress-induced Cort concentrations than less neophobic birds ($F_{1, 24}$ = 3.40, p = 0.079; Fig. 1e). Stress-induced Cort was significantly associated with exploratory tendency ($F_{1, 24}$ = 5.04, p = 0.035; Fig. 1f), with more exploratory individuals having lower stress-induced Cort concentrations than slow explorers.

State-dependent and permanent inter-individual differences

Mass at capture did not differ between age classes (adult mean = 11.24 ± 0.11g, juvenile mean = 11.15 ± 0.11g; GLM $F_{1,64}$ = 0.38, p = 0.54) neither did condition at capture (GLM $F_{1,64}$ = 0.66, p = 0.43). At capture, males weighed more (male mean = 11.33 ± 0.09g, female mean = 10.89 ± 0.09g; GLM $F_{1,67}$ = 6.12, p = 0.016) and were in better condition (GLM $F_{1,66}$ = 6.46, p = 0.013) than females. Birds

that were in poor condition upon capture put on significantly more mass in captivity than those in better condition (Kendall's tau = -0.32, N = 68, $p < 0.0001$).

Following GLM modelling to investigate the relationship(s) between baseline Cort concentrations and state-dependent traits, all variables dropped out of the model ($p > 0.2$) except the interaction between age and change in mass (GLM $F_{1, 35} = 5.92$, $p = 0.021$; age $F_{1, 35} = 2.76$, $p = 0.11$; change in mass $F_{1, 35} = 0.36$, $p = 0.55$; Fig. 2). Baseline Cort was lower in juveniles that increased in mass whilst in captivity than compared with those that lost mass, with the opposite relationship in adults.

When the above model was re-run with stress-induced Cort as the dependent variable; change in mass ($F_{1,28} = 5.26$, $p = 0.030$; Fig. 3a) and haematocrit level ($F_{1,28} = 5.40$, $p = 0.044$; Fig. 3b) remained in the GLM. Across all individuals, birds that put on more mass in captivity, typically those that were in poor condition at capture, had higher stress-induced Cort concentrations than those that lost mass in captivity. Also, birds that had lower haematocrit levels (i.e. were more anaemic) had higher stress-induced Cort concentrations than those with higher haematocrit scores.

Change in mass and haematocrit contributed additively to the model and were not correlated with one another (Kendall's tau b = - 0.13, N= 65, $p = 0.14$). Age, sex, condition at blood sampling and feeding bout duration (an index of hunger and motivation to feed on day 1) dropped out of the model.

Discussion

In this study, we investigated extrinsic and intrinsic correlates of baseline and stress-induced plasma Cort concentrations in wild blue tits held under standard conditions. Both baseline and stress-induced Cort concentrations varied widely despite all animals being maintained at a standard temperature with *ad libitum* food availability for the 24 hours prior to blood sampling. Weather on the day of capture, which might indicate the condition of birds on entry to the study, was not related

to Cort. Notably, neophobia and exploratory tendency, although significantly repeatable within individuals, at least over a 2-3 day interval, did not predict and were not related to baseline Cort concentrations. There was a hint that birds that were generally more active in the exploration trial had lower baseline Cort than less active individuals, but this was driven by data from a small number of females. Unfortunately, given the small number of females sampled, we cannot interpret this sex-difference further. With stress-induced Cort, more exploratory individuals had lower Cort concentrations than less exploratory birds. In contrast, there was a tendency for more neophobic birds to exhibit lower stress-induced Cort than less neophobic birds. There were also relationships between Cort and individual state. Variation in baseline Cort concentrations was determined by a significant interaction between age and change in mass in captivity. Juveniles that had gained mass in captivity had a lower baseline Cort concentration than those that declined in mass. Conversely adults that put on more mass in captivity had higher baseline Cort than those that lost mass. With stress-induced Cort though, all birds that put on mass in captivity had higher stress-induced Cort concentrations than those that lost mass, although condition at blood sampling did not contribute significantly to the model. Birds with a low haematocrit level also had higher stress-induced Cort concentrations than birds with higher haematocrit levels.

Our mixed evidence for associations between behavioural responses to novel and potentially stressful situations and physiological responses to stress are intriguing but not without precedent (Carere et al., 2003; Martins et al., 2007; Scheid & Noe, 2009; Schoech et al., 2009; Scheid & Noe, 2010). One interpretation of our results is that our behavioural assays did not generate neophobia in blue tits. This was unlikely because most birds took significantly longer to approach the food bowl in the presence of the novel object than in the disturbance phase, although a few individuals were clearly not neophobic, approaching the food faster in the presence than absence of the novel object. This is consistent with the notion that the object created a motivational conflict between desire to feed and to avoid the object which is perceived as a risk (Mettke-Hofmann et al., 2002). However, whether the novel objects induced ‘stress’ is harder to determine. The classic flight

or fight response to acute stress, is typically (Buchanan, 2000) but not always (Muller et al., 2006) associated with an elevation in Cort in wild animals. The few studies on birds comparing Cort before and after presentation of a novel object, or more broadly Cort concentrations in high neophobic versus low neophobic individuals, find different responses depending on the experimental design. Generally, those placing a novel object with food identify a Cort response but those presenting the novel object in a neutral location do not (Daisley et al., 2005; Martins et al., 2007; Tobler & Sandell, 2007; but see Apfelbeck & Raess, 2008). This absence of a Cort response (although of course other stress hormones might have been elevated) in neutral studies comes even when accompanied by typical "stress" behaviours – e.g. aversions, tonic responses, distress call (Daisley et al., 2005). Furthermore, within the same species, latency to approach a novel object in a neutral location is not necessarily correlated with latency to approach a novel object with food, suggesting they stimulate different behaviours (Mettke-Hofmann et al., 2002). However, stereotypical stress behaviours are not necessarily evidence of physiological stress (Muller et al., 2006). Neophobia, i.e. the avoidance of novelty, might be a behavioural strategy to avoid a potentially stressful situation. Therefore, we cannot assume that neophobia is an index of responsiveness to stressors in general. Neophobia is simply a behavioural response to something unfamiliar which may or may not be dangerous or stressful. Indeed, contrary to predictions, we found a tendency for more neophobic birds to show a lower hormonal stress response than low neophobia birds, but this needs to be interpreted with caution. Moreover, we also have the issue that we might have captured a biased sample of the least neophobic individuals in the population. Certainly we caught fewer females than males which might suggest that we were lacking data on individuals who were subordinate at feeders, but also potentially those that avoided novel food sources in the wild. The ecological significance of neophobia in the wild is still to be fully assessed.

Exploration has been previously shown to be linked with stress-induced Cort in some but not all species studied (reviewed in Cockrem, 2007), although interpretation of the patterns can be complex. For example, in captive populations of passerines subject to selection on either

behavioural or hormonal responses to stressors, Cort and behaviour are sometimes related: In two lines of great tits (*Parus major*) selected to be either fast or slow explorers, Carere et al. (2003) found that slow exploring birds had higher stress-induced Cort metabolites in their faeces and less impairment in activity than fast birds when stressed (Carere et al., 2003). In zebra finches (*Taeniopygia guttata*), over five generations researchers selected for high or low stress-induced Cort. In the low Cort line, stress-induced Cort concentration was positively correlated with exploratory behaviour but no relationship was present in the high Cort or control lines (Martins et al., 2007). Thus, the results were ambiguous, but provide some evidence of heritable coping styles suggesting selective maintenance of variation in Cort secretion patterns within populations. In contrast to these studies in which the measurement of exploration and activity were confounded (Carere et al., 2003; Martins et al., 2007) we found no relationship between exploration and baseline Cort. On the contrary, experimentally increasing circulating Cort can increase activity levels in birds (Breuner et al., 1998). Thus we suggest that exploratory tendency *per se* is not a behavioural indicator of chronic stress, or that individuals that are chronically stressed are less likely to gather information about a novel environment. Comparison of our behavioural results with those of others (Marchetti & Drent, 2000; van Overveld & Matthysen) suggests that interpretation of "exploratory tendency", and particularly its importance in explaining differences in variation in information acquisition and use, is dependent on the method by which exploration is scored. The differing patterns of covariation between circulating Cort concentrations and personality traits suggests that the physiological mechanisms and potentially the costs of different personality traits will vary. Thus, we would emphasize the need to check the assumptions that exploration, activity and neophobia / risk responsiveness are always correlated or that they represent three measures of the same trait (Reale et al., 2007).

One issue to be explored is the impact of bringing birds into captivity on baseline Cort measurements and both behavioural and hormonal stress responsiveness. In order to standardise the food, temperature and day length experienced by the birds and to ensure that time spent in the

mist net did not influence the baseline Cort measure (Romero & Reed, 2005), we decided to blood sample birds after 24 hours in captivity. However, it might be considered that this reflects a disturbed state. Recent work by Dickens et al (2009) on chukars (*Alectoris chukar*) showed that on the first day of captivity (which involved transport of 100km rather < 3km as in our study) birds lost weight, haematocrit decreased and both baseline and stress-induced Cort concentrations were raised. These effects took 3 to 9 days to fully attenuate. In contrast, we found that after 24 and 48 hours of captivity, the majority of blue tits had gained rather than lost body mass. Moreover, the plasma concentrations of Cort we measured in captivity were similar to those measured in the field at the capture site (basal Cort in the field in 2008 only: 3.2 ng/ml ±1.8, n = 36; in captivity: 4.2 ng/ml ±3.1, n = 16, t-test P = 0.22; (Henderson et al., 2013)). Thus, the elevated stress markers seen in chukars (Dickens et al., 2009) might have been associated with the long distance transport, during which animals often do not feed or drink, rather than captivity *per se*, as has been observed in livestock (Fazio & Ferlazzo, 2003). More research is needed to determine whether individuals with different personality traits vary in how they cope with captivity. It should be noted that we have previously shown that neophobia and exploratory tendency measured in the wild have been shown to be consistent over several weeks to months and was predicted by measurements taken in captivity using analogous methods and the same individuals (Herborn et al., 2010). Thus, whilst only reflecting a snapshot in time, we feel that our results have some ecological relevance at least for our study population.

Our data suggest that both baseline and stress-induced Cort concentrations in wintering blue tits are associated with state-dependent and permanent variables, as well as personality traits. This is in line with evidence that Cort is involved in maintaining a homeostatic energy balance, allowing individuals to respond to food shortages and environmental perturbations (Wingfield & Kitayskya, 2002; Bonier et al., 2009; Hau & Goymann, 2015). For adults only, in the case of baseline Cort, and birds (independent of age), in the case of elevated Cort, birds that put on more mass in captivity had higher plasma Cort concentrations. Moreover birds that were light on entry into

captivity gained more mass during captivity than relatively heavy individuals. What we do not know, and could only find out with manipulative experiments, is whether the poor quality birds gained mass because of their high Cort concentrations (which can stimulate hyperphagia (Landys et al., 2004)) or the rapid increase in mass resulted in raised baseline and stress-induced Cort. There was no sex difference in the relationship between basal Cort and change in mass, although on entry into captivity males were in better condition than females. In contrast to adults, for juveniles the relationship between baseline Cort and mass gain was negative. However, juveniles and adults did not, on average, differ significantly in mass upon capture or their mass change in captivity. Thus, juveniles may have a different stress physiology and/or feedback mechanisms from adults (Bonier et al., 2009). Experience in coping with environmental perturbations and/or relative dominance status have also been shown to influence how birds respond to changes in food supply (Gosler, 1996; Grieco et al., 2002), and thus potentially Cort levels.

For stress-induced Cort, high concentrations were associated with low haematocrit (indicating poor condition) and a greater mass increase. These data indicate that high Cort may signal individuals in poor condition – which might be true for both basal and stress-induced Cort. However, this needs further study, not least because one limitation of our study is that we did not have basal and stress-induced Cort from the same individual for welfare reasons. Thus, we do not know whether blue tits with high basal Cort also had high stress-induced Cort (which might actually indicate a relatively small change in Cort in response to stress) or those with low basal Cort could have high stress-induced Cort (i.e. had a relatively great physiological response to restraint). Selection experiments have shown that basal and stress-induced Cort levels are not necessarily linked (Martins et al., 2007), but this requires more investigation in relation to behavioural responses.

To conclude, exploratory tendency, activity and neophobia in blue tits were uncorrelated and showed differing patterns of co-variation with state-dependent, permanent and physiological traits. As individuals vary along these three independent axes of behavioural variation, the

underlying physiological mechanisms, and indeed the costs (Herborn et al., 2011), may vary in a complex fashion. More research both under captive and wild conditions is needed to determine whether both baseline and stress-induced Cort, i.e. physiological responses to chronic and acute stress, are linked with individual variation in behaviour directly or indirectly via homeostatic control of energetic balance. Of particular interest is how personality traits and other factors that affect ability to control resources interact, and thus influence an individual's current and future energy balance.

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Figure legends

Fig. 1: Relationships between personality traits and plasma Cort concentrations. Baseline Cort concentrations were related to a) activity level in females (crosses, solid line, N = 6), but not in males (circles, dashed line, N = 31); and unrelated to b) exploratory tendency (N = 37); and c) neophobia (N = 35). Stress-induced Cort concentrations were d) unrelated to activity levels (N = 29); e) marginally related to neophobia (N = 24); and f) significantly related to exploratory tendency (N = 29).

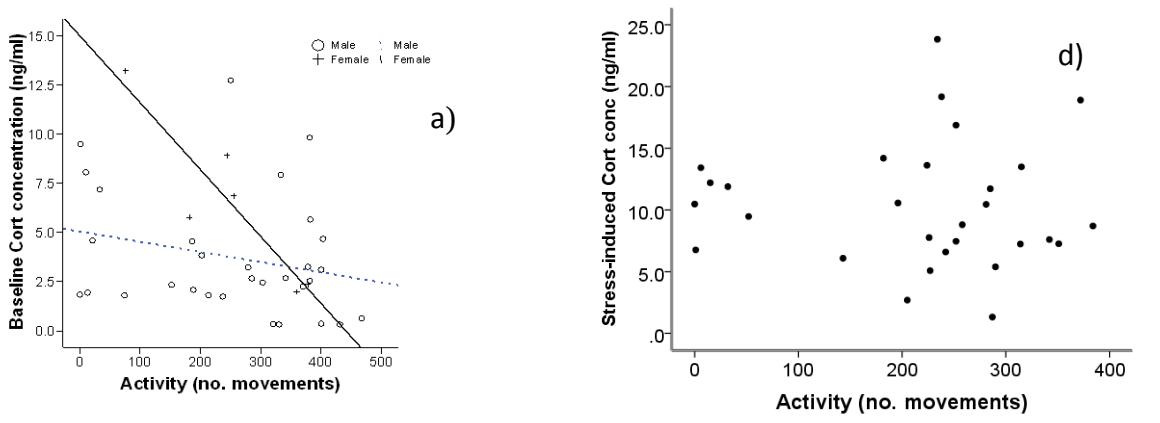
Fig. 2: Relationships between change in mass over two days of captivity (mass at blood sampling on day 2 – mass at capture from wild) and baseline plasma Cort concentrations for juveniles (circles, dashed line, N = 13) and adults (stars, solid line, N = 23).

Fig. 3: Relationships between stress-induced plasma Cort concentrations and a) change in mass over two days of captivity (mass at blood sampling on day 2 – mass at capture from wild)(N = 30); b) Mean haematocrit level (N = 30).

1 Figure 1

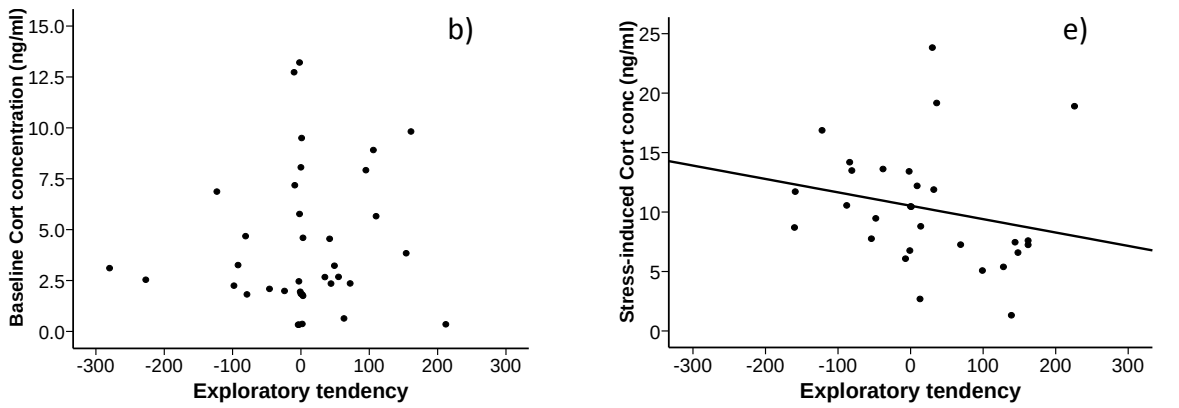
2

3



5

6



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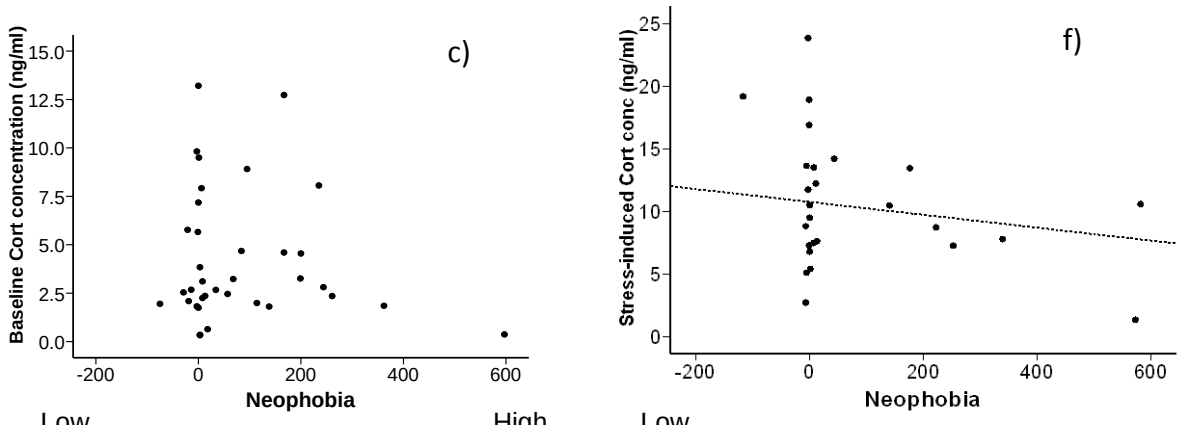


8

9

Figure 2

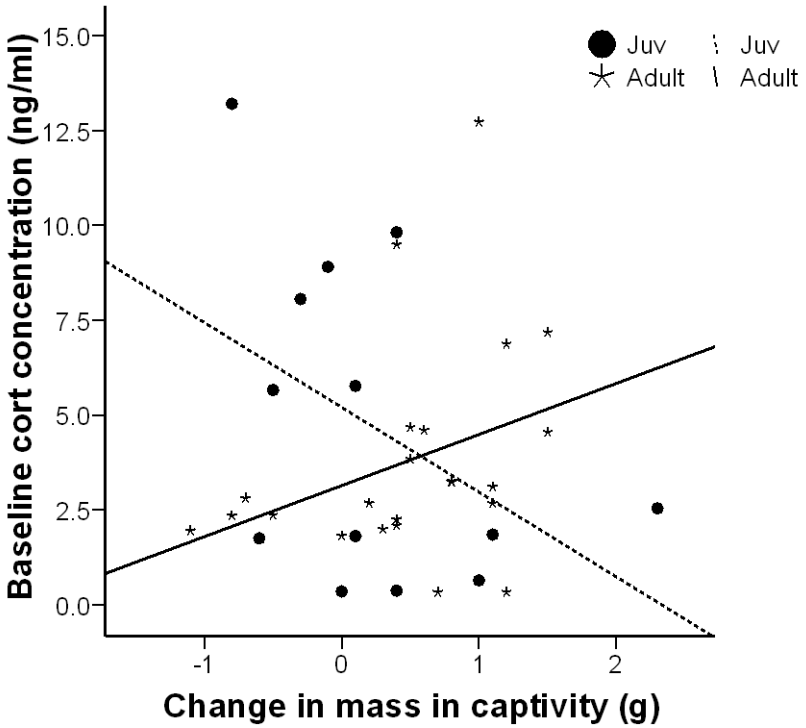
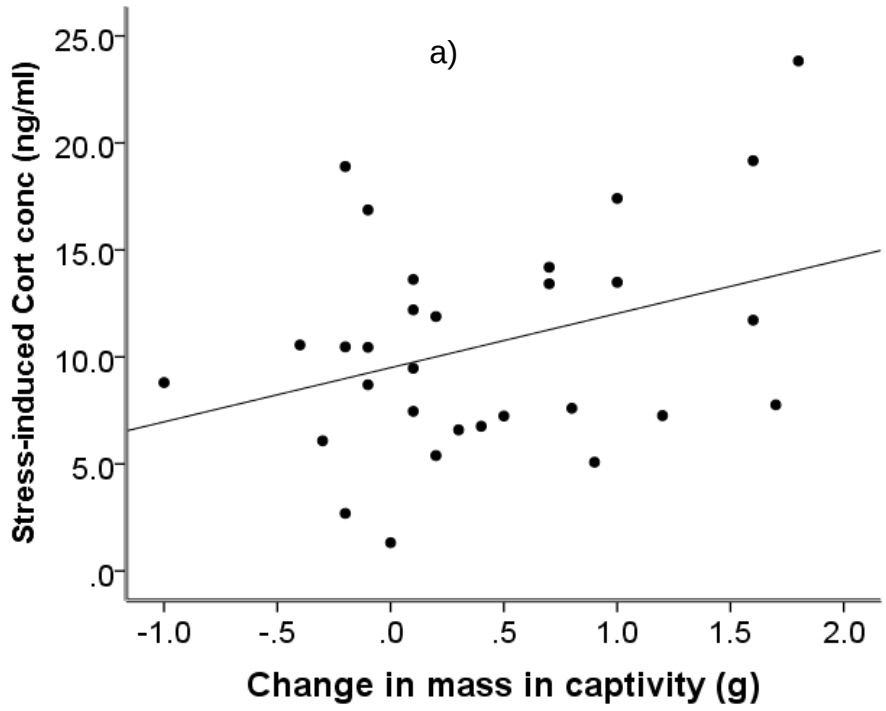
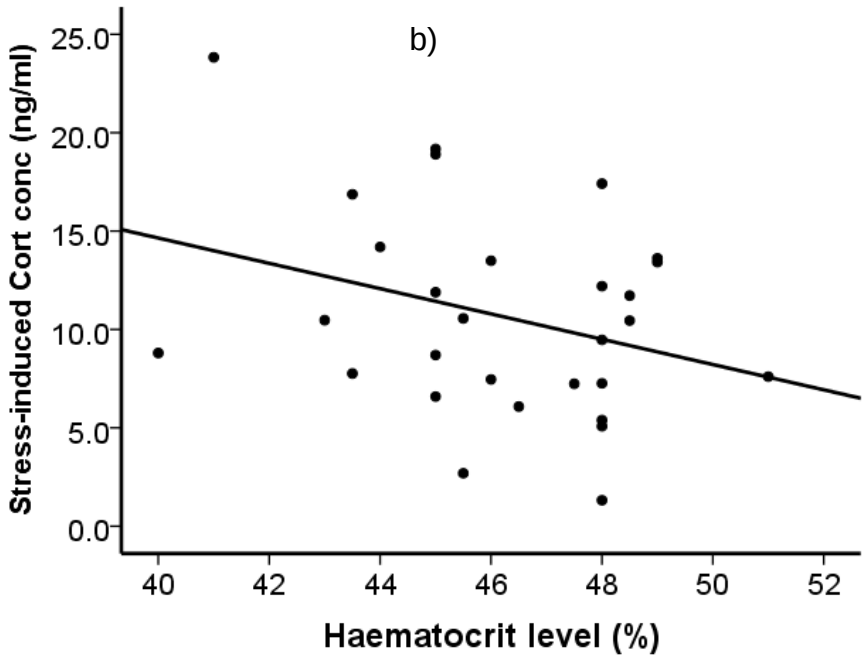

Juv
Adult
Juv
Adult
Baseline cort concentration (ng/ml)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
-1
0
1
2
Change in mass in captivity (g)

Figure 3


a)
Stress-induced Cort conc (ng/ml)
25.0
20.0
15.0
10.0
5.0
.0
-1.0
-.5
.0
.5
1.0
1.5
2.0
Change in mass in captivity (g)


b)
Stress-induced Cort conc (ng/ml)
25.0
20.0
15.0
10.0
5.0
0.0
40
42
44
46
48
50
52
Haematocrit level (%)

SUPPLEMENTARY MATERIAL

(a)

(b)

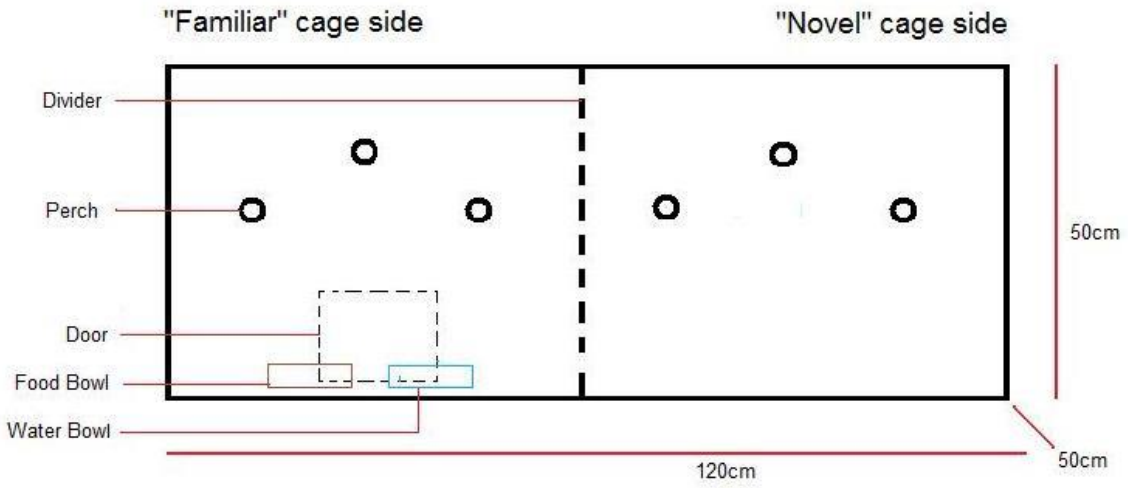
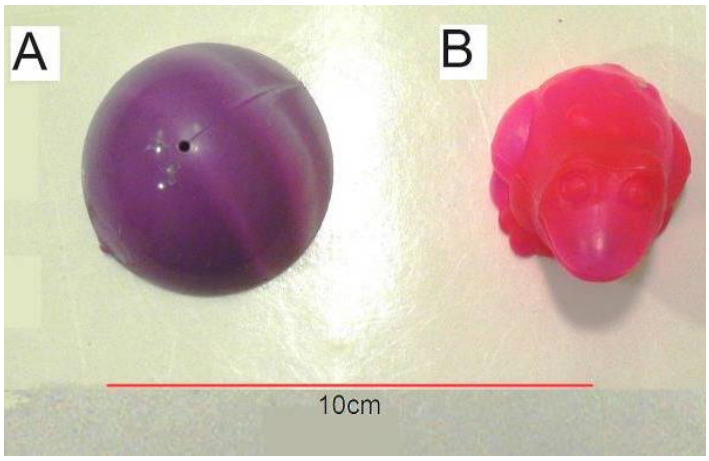


Figure S1: Apparatus used during the captive exploration trials (a) Photo of cage set up. (d) Schematic of cage set up; the food was removed 1hr and water bowl 30min before the start of the trial, via the door.

(a)



(b)

Figure S2: The apparatus used for neophobia trial. (a) A photo of the novel objects used in the neophobia trial: A: half a purple rubber ball, B: a plastic pink frog. (b) A photo of a blue tit approaching the novel object in a food bowl (photo credits: Katherine Herborn).